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ANNUAL PROGRESS REPORT
TO THE
COMMISSION ON ENVIRONMENTAL HYGIENE
OF THE
ARMED FORCES EPIDEMIOLOGICAL BOARD

Sterilization Action of Chlorine and Iodine on
Bacteria and Viruses in Water Systems

For the period: 1 March 1964 to 28 February 1965

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Annual report to the Commission on Environmental Hygiene
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1) Effect of Iodine on RNA Bacterial Virus (f2)

The inactivation of the bacterial virus f2 by iodination is greatly influenced by the presence of the iodide ion, upon the pH and temperature. The inactivation of the f2 phage is limited to about 90% of initial PFU/ml when the iodine to iodide is increased to a ratio of 1 to 100. When the iodine to iodide ratio is 1 to 1.2 or less phage is rapidly inactivated to almost completion (99.999%) in waters at 30°C and pH 7.0 or higher, whereas only 99.9% reduction is obtained in water of pH 7.0 or higher at 5°C. The extent and rate of inactivation is much reduced in waters of pH less than 7.0. Therefore, waters disinfected with the existing globuline tablet may inactivate enteric viruses at a significantly reduced rate especially in cold water areas.

2) Infectious RNA from Iodine Inactivated Phage

The infectious RNA activity is completely resistant to treatment with iodine. Concentration of 100 ppm of iodine applied for 10 minutes to phage yielded infectious RNA in quantities indistinguishable from that of normal phage.

7. Key words: water disinfection, water supply
halogenation
iodination
deoxyribonucleic acid
virus, RNA bacteriophage

INTRODUCTION

This, the third annual progress report on studies of the mode of action of the halogens in the inactivation of bacteria and viruses will largely conclude the work on the action of iodine on bacterial virus. In previous reports it was shown that, due to the sensitivity of the sulphhydral group to iodine, the disinfection of bacteria is assured over a wide range of anticipated conditions of water supply. In the inactivation of bacteriophage, however, it was found that the iodide ion concentration in a reaction mixture significantly influences the action of iodine. Furthermore, the presence of large amounts of organic matter in water systems will convert iodine to iodide and retard the viricidal action of iodine. The disinfection of bacteriophage with iodine is best at high values of pH and poorest in waters below pH 6.0 which may be attributed to the behavior of the iodination of tyrosine in the protein coat of the virus particle.

The iodine tablet (or globuline tablet) employed by the Armed Forces has the following composition by weight (1):

<u>Ingredient</u>	<u>Minimum weight, mgms</u>	<u>Maximum weight, mgms</u>
Tetraglycine hydroperoxide	19.3	21.3
Sodium acid pyrophosphate	82.5	92.0
Inert material	--	6.0

According to Morris, et al. (2) sodium acid pyrophosphate (disodium dihydrogen pyrophosphate) was employed as an active ingredient in the iodine tablet "because it imparts desirable physical properties to the tablet and its acidity serves to lower the pH of natural waters and thus prevent hydrolysis of the elemental iodine into less germicidal substances."

The use of the globuline tablet in water disinfection will tend to lower the pH of the reaction mixture and therefore the inactivation of virus in the lower ranges of pH was studied in detail. Experiments currently reported involve the rate of inactivation of the f2 bacteriophage by iodine at high and low pH and at temperatures of 5° and 30° centigrade.

Considerable time and effort has gone into the study of the effect on the infectious RNA derived from iodine disinfected virus. Work with the f2 bacteriophage has now been completed and details of the procedure and results are presented.

MATERIALS AND METHODS

Purification of f2 phage

The f2 strain of bacteriophage was grown in *E. coli* cultures of tryptone media. Three four-liter flasks, each containing two liters of media, were employed to produce a sufficient phage stock. The flasks were incubated at 37°C with vigorous shaking for 3 to 4 hours to yield a virus concentration of 2×10^{11} plaque forming units (PFU) per ml. Cells and debris were removed by centrifugation at 6,000 r.p.m. for 15 minutes on the same day. Three hundred fifty grams of ammonium sulfate were added for each liter of supernatant and stored overnight in the cold room.

The material which precipitated during quiescent settling overnight in the cold room was spun down, taken up in 10 ml of water per liter of original supernatant, adjusted to pH 7 by adding 0.1 N of NaOH, and clarified by centrifugation for 15 minutes at 10,000 r.p.m. in a Sorvall centrifuge. The resulting supernatant was incubated with 2 gamma/ml of RNase and 1 gamma/ml of DNase at 37°C for 1 hour. The phage suspension was again clarified in the No. 50 rotor of a Spinco, Model L preparative centrifuge, at 15,000 r.p.m. for 15 minutes, and the resulting precipitate discarded. The supernatant was centrifuged at 100,000 g (40,000 r.p.m.) for 90 minutes. The resulting pellet was resuspended in water made to a density of 1.4 with CsCl (2.2 g of CsCl per 4 ml of phage solution) and centrifuged in the swinging bucket rotor of the Spinco Model L centrifuge at 35,000 r.p.m. for 20 hours. A phage band in the center of the centrifuge tube was carefully removed, and dialyzed against 0.05 M phosphate buffer containing 0.145 M saline.

Extraction of infectious RNA from bacteriophage f2

To a one ml sample of phage suspension the following mixture was added: 0.3 ml of 0.2 M phosphate buffer, pH 7.2; 0.7 ml of 3 mM ethylenediaminetetraacetate (EDTA), pH 7.2; 0.5 ml of 5% sodium dodecyl sulfate (SDS). The sample was mixed for one minute at room temperature, then shaken vigorously for five minutes with 25 ml of water-saturated phenol (Mallinckrodt Chemical Works; phenol liquefied). After centrifugation at 2,000 r.p.m. for 5 minutes, the aqueous phase was removed and extracted a second time with 2.5 ml of water saturated phenol. Following the second extraction, one ml of the aqueous phase was mixed with 3 ml of 95% ethanol and placed at -20°C to allow the phosphate salts and RNA to co-precipitate. The precipitate was then settled and redissolved in one ml of 3 mM EDTA. The resulting RNA was extracted three times with equal volumes of ether. The residual ether was eliminated by bubbling the nitrogen gas through the RNA suspension.

Preparation of protoplast stock

Cells of *E. coli* C3000 (Hfr) are grown at 36°C with aeration in Brain Heart Infusion (54 g/l; Difco). Twenty ml of the culture media containing 5×10^9 cells/ml are centrifuged. The pellet is resuspended in 0.35 ml of 1.5 M sucrose. The following solutions were added, in order, with gentle mixing after each: 0.17 ml of 30% crystalline bovine plasma albumin (Armour); 0.02 ml of 2 mg/ml lysozyme in 0.25 M Tris (pH 8.1); 0.04 ml of 4% EDTA; and 1.0 ml of PAM medium. After the mixture has been incubated at room temperature for 10 to 15 minutes, 0.2 ml of 10% Mg SO₄ are added to complex the EDTA and stop the reaction. The protoplast stock must be kept cold and used within one hour after preparation.

Method of assaying infectious RNA

Two sets of serial tenfold dilutions of the infectious RNA derived from iodinated and non-iodinated phage were made in 3 mM EDTA, and incubated for 20 minutes at 37°C. Upon completion of the incubation, 0.1 ml of each RNA sample was added to 0.9 ml of protoplast stock. The mixture was incubated for 20 minutes at 37°C, seeded with 0.5 ml of exponentially growing culture of *E. coli* C3000 suspended in 4 ml of PAM media. Two ml volumes of each sample are plated on tryptone agar by the usual soft agar layer procedure, incubated and read for plaque formation.

RESULT

Effect of pH and temperature on rate of bacteriophage inactivation

The following results have been taken in part from an unpublished thesis by Longley (3). It can be seen from the experimental results (Fig. 1) that the survival of the bacterial phage f2 was increased as the pH decreased following iodination at 5°C. As the amount of hydrogen ion increased below pH 6, the rate of phage inactivation was remarkably reduced. At pH 4 to 5, more than 1% of bacteriophage survived after 40 minutes of contact with 0.01 mM (10 ppm) of iodine, while at pH 10, more than 7 log of phage was inactivated within 30 seconds with the same dosage of iodine. The rate of inactivation with varying pH by iodine at 30°C was shown in Fig. 2. The over all rate of inactivation was much greater at 30°C than at 5°C, but there was a consistent pattern in increased survival at low pH below 6.0.

Sanitary engineers are rather wary of disinfection experiments involving the presence of organic matter which may consume the chemical agent intended for the destruction of the test organism. It should be emphasized that the stock suspensions of phage used are quite free from organic substances other than the bacteriophage itself. Increasing the

Figure 1

EFFECT OF pH ON SURVIVAL OF BACTERIAL VIRUS WHEN
TREATED WITH 0.04 mM IODINE AND 0.048 mM IODIDE
TEMP. \sim 5°C

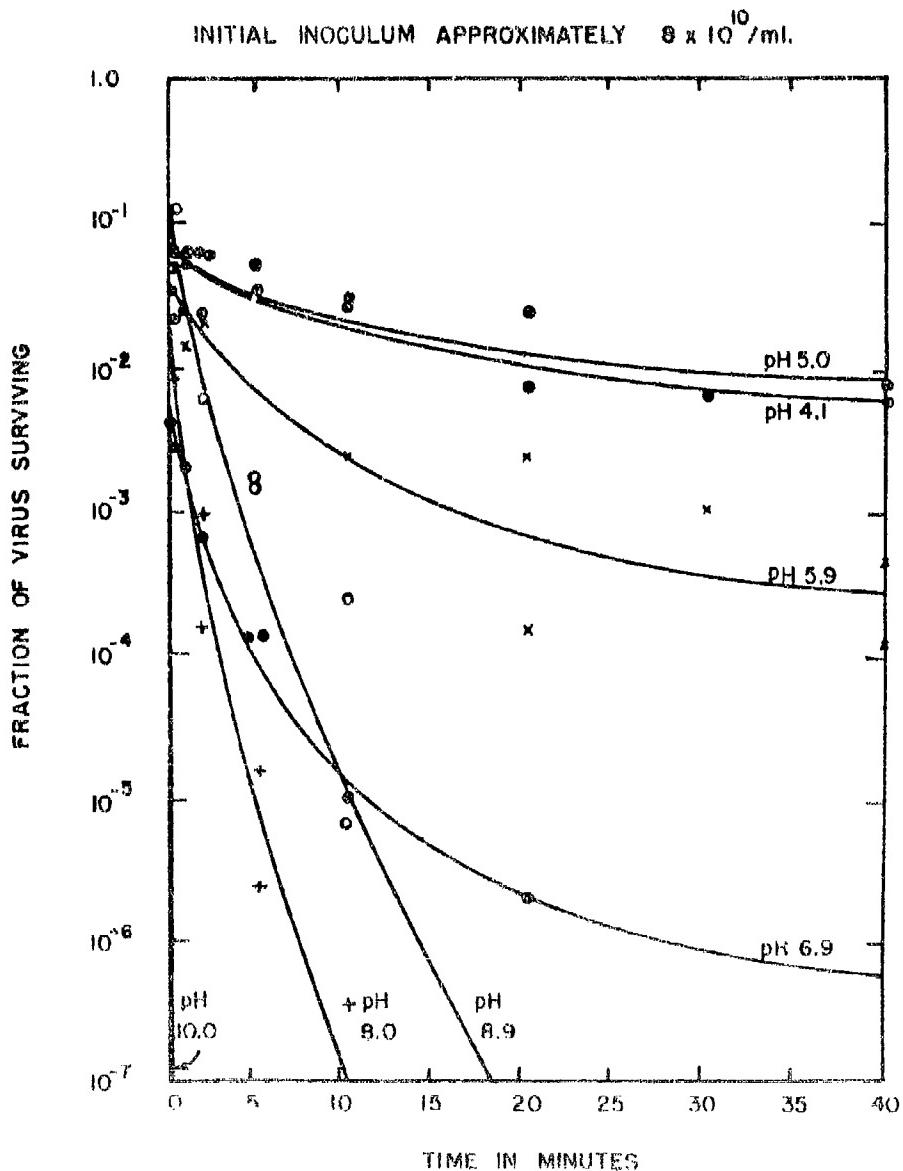
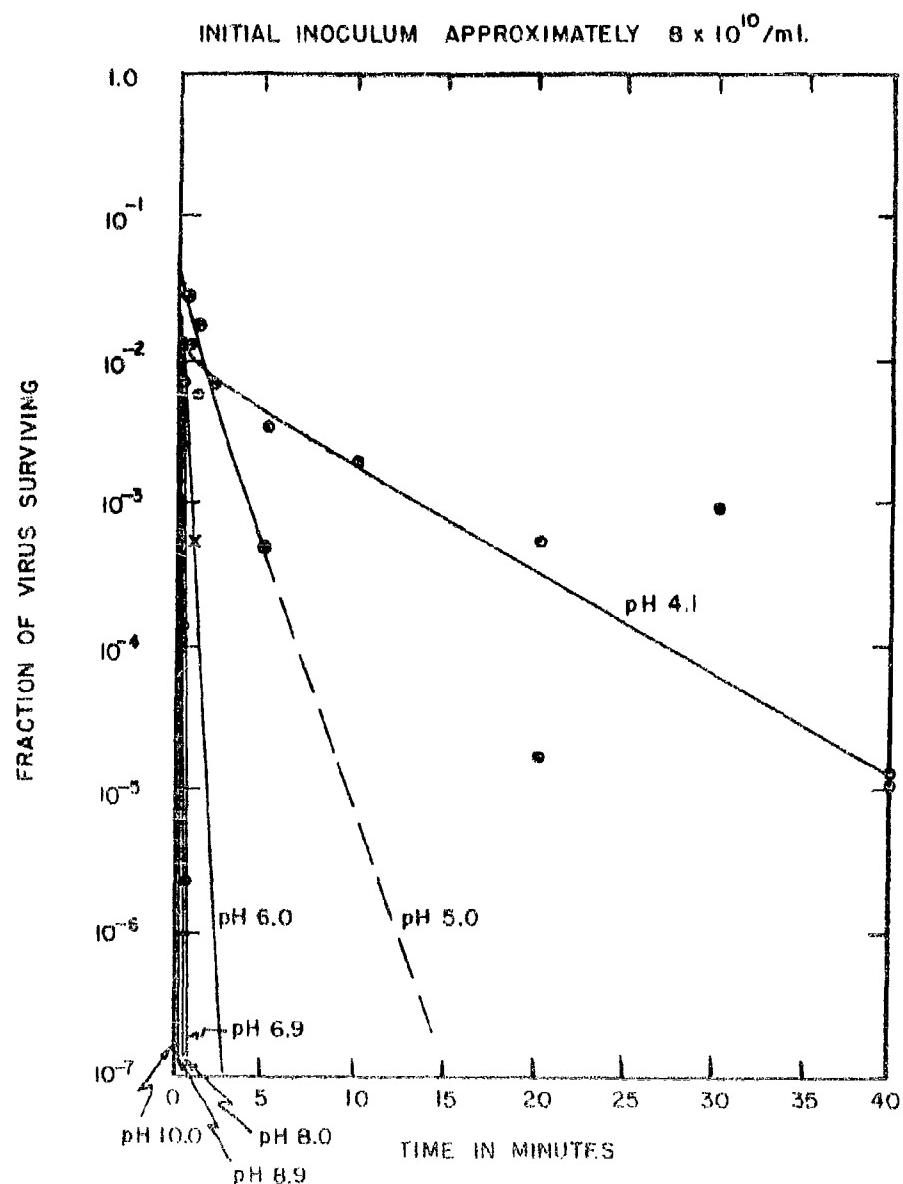


Figure 2

EFFECT OF pH ON SURVIVAL OF BACTERIAL VIRUS WHEN
TREATED WITH 0.04mM IODINE AND 0.048mM IODIDE
TEMP.- 30° C



iodine dosage above 10 ppm does not materially change the phage inactivation end in all tests iodine residuals were observed at the end of contact periods. (It should be recalled that cysts of E. histolytica are destroyed in a wide range of waters and temperatures employing iodine dosages from 8 to 16 ppm.)

The calculated inactivation rate constants as a function of hydrogen ion concentration at 30°C and 5°C are shown in Fig. 3.

Infectious RNA from iodine inactivated phage

Utilizing the above information regarding the most efficient disinfector of phage by iodine, a suspension of f2 phage having 1×10^{13} PFU/ml was treated with 0.2 mM of iodine containing 0.24 mM of potassium iodide for 20 minutes at pH 7.0 and room temperature. The phage survival was negligible with a reduction of 99.9982% or about 5 logs of inactivation.

The infectious RNA was extracted from the iodine inactivated phage and from a similar untreated phage suspension. It was found that the infectivity of the RNA extracted from the treated and untreated preparations was the same. The results are given in Table I, showing that the inactivated phage yield of infectious RNA was in quantities indistinguishable from that of the normal phage.

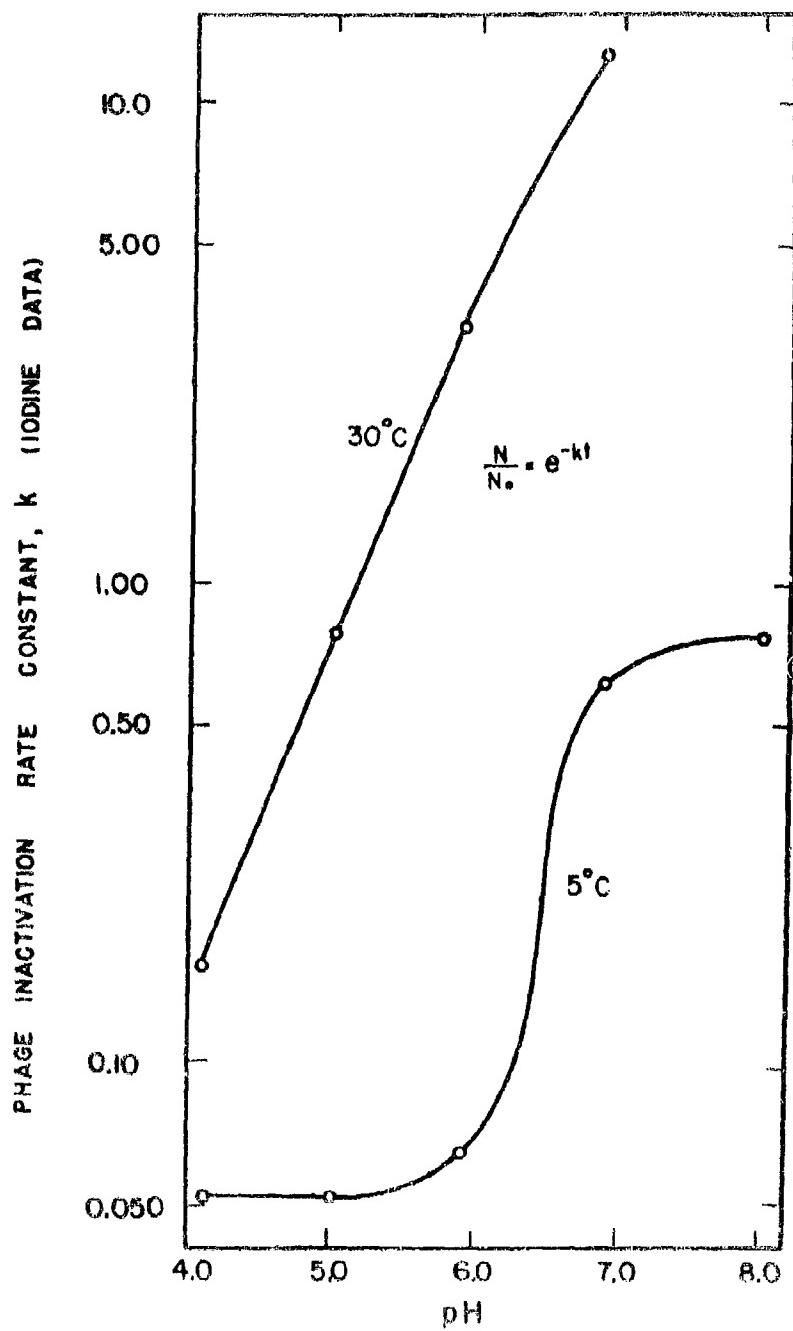
Table I
IODINATION OF INTACT PHAGE AND INFECTIVITY OF ITS RNA

<u>Iodination</u>	Infectivity (PFU per ml)			
	<u>Before Iodination</u>	<u>After Iodination</u>	<u>Survival (%)</u>	<u>Infectious RNA Found</u>
0.2 mM for 20', at room temperature	10×10^{12}	1.8×10^8	0.0018	3.25×10^6
Control	10×10^{12}	1.0×10^{12}	100	3.65×10^6

Iodine resistance of infectious RNA

To test the sensitivity of infectious RNA to iodine, 2.6×10^6 PFU/ml of phage RNA was treated with 101.6 ppm of iodine for 10 minutes at 27°C,

Figure 3
RATE CONSTANT AS A FUNCTION OF HYDROGEN ION CONCENTRATION



thereby guaranteeing a very high iodine residual throughout the entire period of contact. The iodine treated RNA compared with untreated RNA control produced the same amounts of infectious RNA. The results of this experiment are given in Table II and show that the infectious RNA activity is completely resistant to treatment with iodine. There is strong reason to believe that the effect of the halogens on the infectious RNA will parallel previous findings with respect to the destruction of bacterial DNA.

Table II
RESISTANCE OF INFECTIOUS f2 RNA TO IODINE

	<u>Infectivity</u> <u>(PFU/ml)</u>
Iodination by 0.4 mM I ₂ containing 0.48 mM KI at room temperature for 10 min	2.42×10^6
Control	2.55×10^6

SUMMARY AND CONCLUSIONS

The summary and conclusions derived from the third annual progress report are given as the abstract at the beginning.

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Publication during the period covered:

Y. Hsu, Resistance of Infectious RNA and Transforming DNA to Iodine which Inactivates f2 Phage and Cells, Nature 203: 152-153, 1964.